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EVIDENCE FOR A NEW METHYLATED SUGAR: 3-O-METHYL MANNOSE IN THE EXTRACELLULAR POLYSACCHARIDE OF COCCIDIOIDES IMMITIS

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UNITED STATES ARMY
BIOLOGICAL LABORATORIES
FORT DETRICK

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EVIDENCE FOR A NEW METHYLATED SUGAR: 3-O-METHYL MANNOSE IN THE EXTRACELLULAR POLYSACCHARIDE OF *COCCIDIOIDES IMMITIS*.

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ABSTRACT

Large amounts of polysaccharide were obtained from the culture filtrate of the pathogenic fungus Coccidioides immitis grown on a glucose, ammonium lactate, inorganic salts medium. The polysaccharide was precipitated with ethanol, hydrolyzed, and analyzed qualitatively and quantitatively for monosaccharide constituents. Evidence is presented for the identification of the sugars as galactose, glucose, mannose, and 3-O-methyl mannose. 3-O-Methyl mannose has not previously been found in any living organism or as a natural product. The identification of 3-O-methyl mannose is based on the following evidence: demethylation yielded mannose; methoxyl analysis indicated a monomethyl sugar; periodate oxidation at acid pH and 0°C yielded a product that has the chromatographic properties of 2-O-methyl arabinose; and the periodate oxidation product was converted to arabinose by demethylation. Qualitative analysis of the sugar components of the polysaccharide fractions precipitated by increasing amounts of ethanol indicate that there are at least three different polysaccharides in the culture filtrate.

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I. INTRODUCTION

Immunologically active polysaccharides have been isolated from culture filtrates of Coccidioides immitis after prolonged incubation.[1-3] Hassid et al[2] presented evidence for galacturonic acid, glucose, and a third unidentified sugar as components of an isolated polysaccharide. However, Pappagianis[3] cited unpublished data by Putnam, who could not detect galacturonic acid and found mannose to be the major constitutent in hydrolyzates of the polysaccharide preparation of Hassid et al.[2] Pappagianis[3] found mannose and galactose in a polysaccharide fraction he obtained from culture filtrates of C. immitis, strains Silveira and 46.

Previous data by Goldschmidt et al[4] indicated that approximately 3.5 grams of polysaccharide accumulated per liter of culture fluid when C. immitis, strain Cash, was grown in a synthetic medium. Preliminary data suggested that mannose, glucose, galactose, and an unidentified sugar were components of the polysaccharide fraction. This report presents further studies on the polysaccharide with evidence that the unidentified sugar is 3-O-methyl mannose. Results also are cited that suggest that C. immitis culture filtrates contain several different polysaccharides.

II. METHODS

A. PRODUCTION OF POLYSACCHARIDE

C. immitis strain Cash was grown in the glucose, ammonium lactate, inorganic salts medium of Goldschmidt and Taylor.[5] The organism was incubated in six-liter Erlenmeyer flasks containing 500 milliliters of medium on a reciprocating shaker at 34°C for seven days.

B. PREPARATION OF POLYSACCHARIDE

The cultures were sterilized by adding merthiolate at a final concentration of 0.1 per cent and incubating at room temperature for several hours. Sterility was determined by plating one milliliter of culture in pour plates of an agar medium used for viable count determinations of C. immitis.[5] After sterility was ascertained, the cultures were filtered on a Buchner funnel with washed Celite 535 and the combined filtrates (three liters) concentrated by flash evaporation to one-tenth the original volume. The polysaccharide was precipitated by adding four volumes of ethanol except when stated otherwise. The precipitate was dissolved in water and dialyzed against distilled water overnight at 4°C. The polysaccharide was reprecipitated with ethanol, washed with absolute ethanol, a 1:1 mixture of absolute ethanol and diethylether, and finally with ether, and dried in vacuo over calcium chloride.

C. ANALYTICAL TECHNIQUES

Total carbohydrate was determined with the anthrone method of Seifter et al.[6] Reducing sugar was determined according to Somogyi,[7] using the arsenomolybdate reagent of Nelson[8] for the colorimetric procedure. Both determinations were expressed in terms of glucose with a chromogen equivalent of 100 per cent per mole. Different sugars varied with respect to the amount of chromogen produced by the two procedures. The reducing sugar procedure yielded chromogen equivalents of: galactose, 77.4 per cent; mannose, 76.3 per cent; and 3-O-methyl mannose, 30.2 per cent per mole. However, the anthrone procedure yielded equivalents of: galactose, 54.3 per cent; mannose, 48.1 per cent; and 3-O-methyl mannose, 42.7 per cent per mole. The results with 3-O-methyl mannose were based on the assumption that the sugar was not hydrated.

Periodate oxidation at pH 7.5 to determine the yield of formaldehyde was performed by the procedure of Metchell and Percival.[9] Formaldehyde was assayed colorimetrically as described by Lambert and Neish.[10] Determination of the substituent position of the methoxyl group was based on the procedure of Lemieux and Bauer.[11]

D. HYDROLYSIS OF POLYSACCHARIDE

The polysaccharide was hydrolyzed in 1 N H_2SO_4 at 105°C for six hours in sealed ampoules. Preliminary experiments indicated that these conditions produced the maximum yield of reducing sugar with the absence of detectable oligosaccharides.[12] The hydrolyzates were neutralized with $BaCO_3$ to the congo red end-point, filtered to remove $BaSO_4$, decolorized with charcoal, desalted by elution from an Amberlite IR-120 (H^+) column, and concentrated by drying *in vacuo*.

E. PAPER CHROMATOGRAPHY

The sugars were chromatographed on paper (Whatman No. 1), using the descending technique with the following solvents: n-butanol-pyridine-water (3:1:1 v/v); n-butanol-ethanol-water (4:1:5 v/v); n-butanol-acetic acid-water (5:1:2 v/v); n-butanol-2,4 lutidine-water (47:30:23 v/v), and phenol-water (80:20 w/v). Results are expressed as R_G (R glucose) values, i.e., the ratio of the mobility of a sugar to the mobility of glucose on the same chromatogram.

Reducing sugars were detected by spraying chromatograms with a modified p-anisidine phosphate spray reagent.[13] The spray reagent was prepared by dissolving 1.5 grams of p-anisidine (free base) in 50 milliliters of 95 per cent ethanol and adding 100 milliliters distilled water and 3.0 milliliters of concentrated H_3PO_4. The chromatograms were heated in a 100°C oven for two minutes to develop the color for quantitative determinations. The colors observed with the different sugars were similar to those observed with aniline phthalate;[14] however, the p-anisidine phosphate reagent was more sensitive. Nonreducing sugars were detected on chromatograms by the $AgNO_3$ procedure of Trevelyan *et al.*[13]

Quantitative determinations of sugars were performed by spraying the chromatogram with p-anisidine phosphate and scanning the chromatogram with the Spinco Analytrol densitometer. Control samples of known concentrations of glucose, mannose, and galactose were analyzed by the same procedure for the preparation of standard curves. There was no significant difference in the integration density of the color obtained with any of these known sugars. The concentrations of the unidentified sugar were calculated on the assumption that the unidentified sugar yields the same color value per mole as glucose.

Demethylation of the unidentified sugar to identify the unsubstituted parent compound was performed by the procedure of Hough, Jones and Wadman.[15]

E. CELLULOSE COLUMN CHROMATOGRAPHY

Pure fractions of each of the four sugars found in the hydrolyzate were obtained by cellulose column chromatography.[16] The hydrolyzate from six grams of polysaccharide was mixed with a small amount of powdered cellulose and transferred to the top of a one-inch-diameter column packed with 60 grams of powdered cellulose. The sugars were eluted with n-butanol-water (20:1) using the time interval method with a Rinco automatic fraction collector. Approximately eight milliliters was collected in each tube and analyzed for reducing sugar with the Somogyi colorimetric procedure. Alternate tubes were analyzed qualitatively for sugars by paper chromatography, using the n-butanol-pyridine-water (3:1:1) solvent. The effluents in tubes that contained the same sugar were pooled and concentrated by flash evaporation and then dried *in vacuo* in a desiccator.

III. RESULTS

A. COMPOSITION OF UNFRACTIONATED POLYSACCHARIDE

Hydrolyzates of the unfractionated polysaccharide were assayed by paper chromatography with the solvent systems indicated in Table I. The results show that the polysaccharide fraction contained four sugars. Three of the sugars were tentatively identified as galactose, glucose, and mannose from their R_G values and color reactions with the p-anisidine phosphate spray reagent. Further studies that support this tentative conclusion and indicate that the fourth sugar is probably 3-O-methyl mannose are presented later in this report.

B. FRACTIONATION OF POLYSACCHARIDE

Fractional precipitation of the polysaccharide with increasing concentrations of ethanol (Table II) indicated that it consisted of a mixture of polysaccharides. Each fraction was assayed for total carbohydrate, hydrolyzed, and the sugar components determined quantitatively by paper chromatography by the densitometer procedure. The results show a heterogeneity in the sugar composition of the different fractions. Fractions precipitated with zero to two volumes of ethanol contained galactose (15.3-18.7 per cent) and low levels of the unidentified sugar. The fractions precipitated at higher ethanol concentrations contained virtually no galactose and seemed to consist of at least two different polysaccharides as shown by the heterogeneity of glucose content. These results may be of significance in future studies concerned with immunologically active polysaccharides produced by C. immitis, since at least three different polysaccharides in the culture filtrate are indicated.

C. IDENTIFICATION OF UNKNOWN SUGAR

Large amounts of the sugars from the hydrolyzed polysaccharides were purified by cellulose column chromatography. The separation of the sugars in one of the chromatography experiments is illustrated in Figure 1. The data show that the unidentified sugar and mannose were eluted as pure fractions, whereas the glucose and galactose fractions were not completely separated from each other. Pure fractions of glucose and galactose were obtained by pooling only those tubes of eluate that contained a single sugar as determined by paper chromatography. Each one of the pooled fractions was compared with known sugars by paper chromatography. The results shown in (Table III) agree with the previous data presented in Table I with reference to the identity of galactose, glucose, and mannose. The fourth sugar is not rhamnose, as shown by the difference in R_G values in the phenol solvent.

TABLE I. PAPER CHROMATOGRAPHY OF HYDROLYZATE OF POLYSACCHARIDE [a] FROM CULTURE FILTRATE

Sugar \ Solvent	n-butanol: 4 ethanol : 1 water : 5	n-butanol : 5 acetic acid: 1 water : 2	n-butanol: 3 pyridine : 1 water : 1	n-butanol : 47 2,4 lutidine: 30 water : 23
Galacturonic Acid	0.19	0.77	0.09	0.05
Glucosamine	0.70	0.68	0.74	0.28
Galactose	0.94	1.00	0.84	0.84
Glucose [b]	1.00	1.00	1.00	1.00
Mannose	1.28	1.34	1.21	1.61
Fructose	1.28	1.41	1.16	1.60
Arabinose	1.30	1.42	1.22	1.62
Xylose	1.44	1.62	1.46	2.02
Ribose	1.72	1.80	1.63	2.39
Fucose	1.71	1.90	1.56	2.08
Rhamnose	2.14	2.16	2.01	3.22
Hydrolyzate: spot 1	0.95 [c]	0.99 [c]	0.85	0.83
spot 2			1.00	1.10
spot 3	1.23	1.23	1.22	1.50
spot 4	1.98	2.04	1.92	2.80

a. Polysaccharide precipitated with four volumes of ethanol and hydrolyzed as described in Section II.
b. All values expressed as $R_{Glucose}$.
c. Glucose and galactose are not separated in this solvent system.

TABLE II. COMPOSITION OF POLYSACCHARIDES FROM SEVERAL ETHANOL FRACTIONATIONS

Ethanol Fractions	Per Cent of Total Carbohydrate [a]	Component Sugars, per cent			
		Galactose	Glucose	Mannose	X [b]
0-1 volume	6.8	18.7	15.7	57.8	7.8
1-2	36.6	15.3	18.5	60.2	6.0
2-3	32.1	2.6	7.3	62.5	27.6
3-4	8.0	0.7	4.9	52.9	41.5
4-5	2.2	0.0	20.7	47.3	32.0
5 volume supernatant	8.5	0.0	41.0	35.2	23.8
Unfractionated polysaccharide	100	9.8	13.0	61.1	16.1

a. Total carbohydrate content determined by the anthrone method.
b. Unidentified component.

TABLE III. R_G VALUES OF SUGARS OBTAINED FROM HYDROLYZATE BY CELLULOSE COLUMN CHROMATOGRAPHY

Sugars \ Solvents	n-butanol: 3 pyridine : 1 water : 1	phenol: 80 gm water : 20 ml	n-butanol: 4 ethanol : 1 water : 5	n-butanol : 5 acetic acid: 1 water : 2
Galactose	0.84	1.10	0.83	0.91
Glucose	1.00	1.00	1.00	1.00
Mannose	1.25	1.17	1.13	1.19
Arabinose	-	1.39	1.13	1.30
Fructose	-	1.32	1.10	1.22
Rhamnose	2.04	1.59	1.98	1.89
4-O-Me·mannose	2.16	1.91	2.05	2.00
Column Fractions:				
"Galactose"	0.84	1.10	0.82	0.91
"Glucose"	1.00	1.02	0.98	1.02
"Mannose"	1.25	1.16	1.12	1.18
"3-O-Me·mannose"	2.00	1.95	1.88	1.85

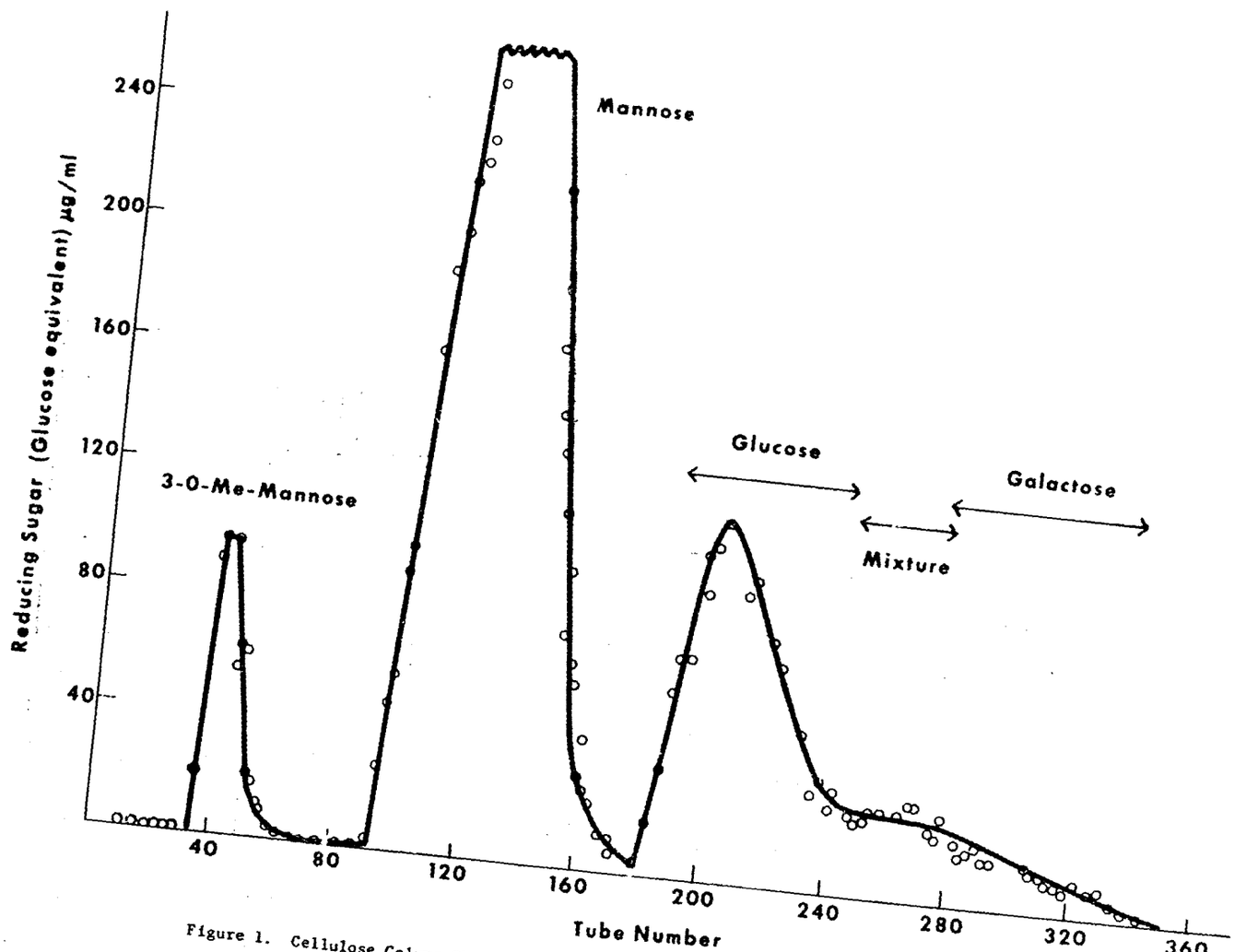


Figure 1. Cellulose Column Chromatography of the Sugar Components of the Extracellular Polysaccharide. The solvent was n-butanol-water (20:1). The sugars were determined by Somogyi's method and were chromatographed with n-butanol-pyridine-water as described in Section II.

The sugars from the hydrolyzate were reacted with phenylhydrazine and the times required for the formation of the derivatives and the microscopic appearance of the crystals were examined.[17,18] The time required for the formation of the mannose phenyl hydrazone, glucose osazone, galactose osazone, and the crystal structures of each were identical with the known sugars.

The mobility of the unidentified sugar on paper chromatograms suggested that it might be a methoxyl derivative. Therefore, it was subjected to the demethylation procedure of Hough, Jones and Wadman[15] and the product chromatographed on paper with several known sugars. The results in Table IV show that the R_G values of the demethylated product in two different solvent systems were identical with that of an authentic sample of mannose.

A methoxyl determination on 7.6 milligrams of the unidentified sugar indicated a methoxyl value of 14.9 per cent by weight. The theoretical value for a mono-methyl hexose would be 16 per cent, or 14.6 per cent if it is assumed that the sugar is the monohydrate form. The latter assumption is probably valid; however, in either case the results suggest that the unidentified sugar is a mono-methoxyl derivative of a hexose.

D. DETERMINATION OF THE SUBSTITUENT POSITION OF THE METHOXYL GROUP

The production of formaldehyde upon periodate oxidation of a methylated hexose at neutral pH is indicative that the methoxyl group is not attached to carbon atoms 5 or 6. Periodate oxidation of a sample of the unidentified sugar produced formaldehyde in yields of 85 per cent of the theoretical value assuming that the sugar was a mono-methoxyl derivative of a hexose.

TABLE IV. R_G VALUE OF DEMETHYLATED PRODUCT OF UNIDENTIFIED SUGAR

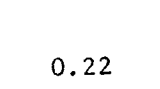
Solvent / Sugars	R_G Value: n-butanol : pyridine : water 3 : 1 : 1	R_G Value: phenol : water 80 gm : 20 ml
Glucose	1.00	1.00
Galactose	0.84	1.14
Mannose	1.18	1.23
"Demethylated Product"	1.17	1.25

Lemieux and Bauer[11] described a procedure for the identification of the different mono-O-methyl glucoses. This procedure is based on the fact that oxidation with periodate at pH 2.0 to 4.8 at 0°C yields a stable formyl ester from the lactol carbon atom (oxidation between carbon atoms 1 and 2). This formyl ester blocks further periodate oxidation and serves as a unique identification product for each of the different mono-O-methyl glucoses. After destruction of the excess periodate, the formyl group is removed by neutralization to the phenolphthalein end-point and the periodate oxidation product is identified by paper chromatography. The results of this determination with the unidentified sugar are shown in Table V. The mobility (R_f) and the color of the spot suggest that the reaction product was 2-O-methyl arabinose.

Another chromatogram was used to prepare a large amount of the periodate oxidation product. The product was eluted from the chromatogram and demethylated with HBr as described previously. The demethylated product was identified as arabinose upon comparison of the R_G value and the color reactions of the spots obtained upon paper chromatography of the unknown sugar and comparison with authentic specimens of ribose, xylose, and arabinose in two different solvent systems (n-butanol-pyridine-water and phenol-water). These results also indicate that the periodate oxidation product was 2-O-methyl arabinose.

The results of this periodate oxidation procedure suggest that the unidentified sugar is 3-O-methyl mannose.

TABLE V. CHROMATOGRAPHY OF PERIODATE OXIDATION PRODUCTS OF O-METHYL DERIVATIVES OF HEXOSES

Hexose	Product	R_f	Color - Aniline Phthalate Spray
Theoretical:			
2-O-methyl glucose		0.22	lemon yellow
3-O-methyl glucose	2-O-methyl arabinose	0.35	plum
4-O-methyl glucose	2-O-methyl erythrose	0.53	citrine
6-O-methyl glucose	3-O-methyl glyceraldehyde	0.71	canary yellow
Experimental:			
unidentified sugar	—	0.37	plum
4-O-methyl mannose	2-O-methyl erythrose	0.44	orange
2,5 dimethyl mannitol	2-O-methyl glyceraldehyde	0.72	yellow

Periodate oxidation products of O-methyl glucose derivatives from Lemieux and Bauer.[11]
R_f-Solvent n-butanol-ethanol-water (4:1:5)

IV. DISCUSSION

The identification of the unknown sugar obtained from the hydrolyzate is based on the following results. The mobility of this sugar on paper chromatograms with different solvents was similar to that of 4-O-methyl mannose. When the sugar was demethylated, mannose was obtained as a product. The methoxyl determination indicates that the sugar is a mono-methoxyl derivative of a hexose. Periodate oxidation at neutral pH yielded formaldehyde, which eliminates the five or six methoxyl derivatives of mannose as the correct structure for this unknown sugar. Periodate oxidation at acid pH and 0°C yielded a compound that had the same mobility and color reaction as 2-O-methyl arabinose. Demethylation of the periodate oxidation product yielded arabinose, which confirms the previous result that 2-O-methyl arabinose was the oxidation product. Only the 3-O-methyl derivative of mannose should yield 2-O-methyl arabinose as the periodate oxidation product.

All of these results are in agreement with the identification of this sugar as 3-O-methyl mannose. However, until this sugar is crystallized and the physical properties are shown to be identical with known 3-O-methyl mannose, and derivatives are prepared that also show identical properties with those of the same derivatives of the known sugar, the identification must be regarded as tentative.

In 1956, Aspinall[80] reviewed the properties of the methyl ethers of mannose. Later, in 1957, he reported the synthesis and characterization of 3-O-methyl mannose.[80] However, we have not seen any data on the occurrence of this sugar in natural products.

Several different O-methyl derivatives of sugars have been found in natural products.[21-23] These methylated sugars have been found in the cardiac glycosides,[21] plant gums and hemicelluloses,[21,22] Streptomyces antibiotics,[22] and the glycolipids of Mycobacterium tuberculosis.[23-26]

Further studies are necessary to determine the immunological properties of these polysaccharides. The results indicate that large quantities of these polysaccharides can be prepared by growth in a synthetic medium and future studies should assess what immunological functions they possess.

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